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Sequence variation in trypsin- and chymotrypsin-like cDNAs from the midgut of *Ostrinia nubilalis*: methods for allelic differentiation of candidate *Bacillus thuringiensis* resistance genes

B. S. Coates*†‡, R. L. Hellmich*‡ and L. C. Lewis*‡

**USDA-ARS, Corn Insects and Crop Genetics Research Unit; †Interdepartmental Genetics Program; and ‡Department of Entomology; Iowa State University, Ames, IA, USA*

Abstract

Midgut expressed alkaline serine proteases of Lepidoptera function in conversion of *Bacillus thuringiensis* (Bt) protoxin to active toxin, and reduced level of transcript T23 is associated with *Ostrinia nubilalis* resistance to Dipel® Bt formulations. Three groups of trypsin- (OnT25, OnT23, and OnT3) and two chymotrypsin-like (OnC1 and OnC2) cDNAs were isolated from *O. nubilalis* midgut tissue. Intraspecific groupings are based on cDNA similarity and peptide phylogeny. Derived serine proteases showed a catalytic triad (His, Asp, and Ser; except transcript OnT23a), three substrate specificity-determining residues, and three paired disulphide bonds. RT-PCR indicated all transcripts are expressed in the midgut. Mendelian-inherited genomic markers for loci OnT23, OnT3 and OnC1 will be useful for association of alleles with bioassayed Bt toxin resistance phenotypes.

Keywords: Ostrinia nubilalis, serine proteases, Bacillus thuringiensis resistance.

Introduction

Transgenic crops that express insecticidal toxin proteins derived from *Bacillus thuringiensis* (Bt) reduce or eliminate feeding by pest insects (Crickmore *et al*., 1998; Walker *et al*., 2000). The Cry1Ab gene was incorporated into corn (Koziel *et al*., 1993) and other crop plant germplasm (Frutos *et al*., 1999). Commercial corn hybrids expressing Cry1Ab toxin have been available since 1996 (Rice & Pilcher, 1998) and in 2004 were planted on 29% of U.S. corn ground (22.9 million ha; USDA-NASS, 2004). Transgenic Cry1Ab coding sequences have been truncated (3′ and 5′) and codons altered from the native *B. thuringiensis* ssp. *kurstaki* gene (Vaeck *et al*., 1987), which might affect crystallization or proteolytic activation requirements by endogenous proteases in susceptible insect midguts (Schnepf *et al*., 1998; Rukmini *et al*., 2000).

Moderate *Ostrinia nubilalis* resistance to Cry toxins was observed after laboratory selection (Bolin *et al*., 1999; Huang *et al*., 1999b; Chaufaux *et al*., 2001), and frequency estimates of resistant phenotypes in natural populations are $\leq 9.2 \times 10^{-4}$ (Andow *et al*., 1998; Andow *et al*., 2000; Bourguet *et al*., 2003). Reduced expression of a major trypsin protease in midgut tissue was shown for *Plodia interpunctella* phenotypes resistant to CRY1Ab (Oppert *et al*., 1996; Zhu *et al*., 2000). Midgut extracts from the *O. nubilalis* KS-SC strain, reared on Dipel® Bt formulation, had reduced trypsin-like protease activity and 35% decreased capacity to hydrolyse CRY1Ab protoxin compared to susceptible controls (Huang *et al*., 1999a). Li *et al*. (2004) showed a reduced rate of toxin activation in some *O. nubilalis* strains was correlated with decreased trypsin-like protease activity. Li *et al*. (2005) further demonstrated a 2.7–3.8-fold reduction in trypsin T23 transcript level in Dipel® resistant compared to susceptible individuals.

Families of serine protease genes are expressed in the epithelial cells of insect midguts (Peterson *et al*., 1994; Brown *et al*., 1997). Trypsin-like proteases cleave following highly basic residues (Arg and Lys), and are determinants of *B. thuringiensis* protoxin activation (Haider & Ellar, 1989; Oppert *et al*., 1997; Rukmini *et al*., 2000; Li *et al*., 2004; Li *et al*., 2005). Chymotrypsin-like proteases cleave following aromatic residues (Trp, Tyr, and Phe), and may degrade active (trypsinized) Cry toxins, rendering them non-toxic to

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Received 6 April 2005; accepted after revision 15 July 2005. Correspondence: Brad S. Coates, USDA-ARS, Corn Insects & Crop Genetics Research, 113 Genetics Laboratory, Iowa State University, Ames, IA 50010, USA. Tel.: +1 515 2940 668; fax: +1 515 2942 265; e-mail: coates@iastate.edu

susceptible insects (Yamagiwa *et al*., 1999; Miranda *et al*., 2001). *Culex pipiens* chymotrypsins were implicated in breakdown of active CRY4A toxins (Yamagiwa *et al*., 1999). Native CRY1Ab tolerance of *Spodoptera frugiptera* might lie with its high active toxin degradation rate compared to more susceptible *Manduca sexta* larvae (Miranda *et al*., 2001). Although not implicating chymotrypsins, Keller *et al*. (1996) also showed increased rates of Cry1C toxin degradation in late instar *Spodoptera litoralis* was due to enhanced serine protease activities.

Midgut serine proteases are part of a CRY1Ab activation pathway, and represent a Dipel® Bt toxin formulation resistance mechanism in *O. nubilalis* (Huang *et al*., 1999a; Li *et al*., 2004; Li *et al*., 2005). An *O. nubilalis* trypsin-like enzyme was isolated by fractionation of midgut juices (Bernardi *et al*., 1996), midgut protease activity properties characterized (Houseman *et al*., 1989), and individual transcripts isolated (Li *et al*., 2005), yet intraspecific variation and molecular markers for these *O. nubilalis* genes have not been described. We report characterization of 18 unique trypsin- and chymotrypsin-like transcripts expressed in midgut of *O. nubilalis*, and represent transcripts from five unique genomic loci. Molecular markers for genomic- and expression-level analysis were developed, and may be used in linkage mapping or studies to associate cosegregation of alleles with bioassayed *O. nubilalis* Bt toxin resistance phenotypes.

Results and discussion

Complementary DNA (cDNA) sequences

Eighteen unique alkaline serine protease-encoding sequences (including two partial sequences; Fig. 1) were observed from 40 clones containing cDNAs from bivoltine Z-pheromone strain 5th instar *O. nubilalis* reared on semi-meridic diet (Guthrie, 1987). All sequences were submitted to GenBank (Table 1). BlastX and Protein Database (PDB) searches (Gish & State, 1993; Rost, 1996) showed high similarity of 11 *O. nubilalis* proteins to trypsin-like peptides and 7 to chymotrypsin-like peptides from other Lepidoptera (GenBank accessions incorporated into phylogeny; Fig. 2). Five trypsin-like cDNAs showed 98–99% similarity to the T25 transcript isolated by Li *et al*. (2005), and were subsequently named OnT25a to OnT25e (Tables 1 and 2). A single transcript OnT23a was 83% similar to the transcript T23 that showed decreased levels in midgut tissue of a Dipel® resistant colony (Li *et al*., 2005). The five trypsin OnT3, and seven chymotrypsin OnC1 and OnC2 transcripts have not previously been characterized from *O. nubilalis*.

Sixteen complete cDNA sequences (omitting two partial sequences OnC1a and OnC2c; Fig. 1) ranged from 821 to 923 nt, with the shortest transcript having a block deletion between consensus codons 44–73 (OnT23a; Fig. 1A). A truncated 156 residue trypsin-like protease, OnT3e, was predicted due to a premature stop codon (TGA), and confirmed by duplicate and overlapping sequencing runs. Two partial chymotrypsin-like cDNA sequences of 677 and 859 nt also were obtained that, respectively, were missing N- and C-terminal coding regions (OnC1a and OnC2c; Table 1). OnC1a and OnC2c may represent cloning artifacts (Brown *et al*., 1997), but were retained within the alignment due to polymorphism they contain. Omitting two partial cDNA sequences and the truncation mutant, cDNA open reading frames ranged from 231 to 291 codons (Fig. 1).

Pairwise sequence similarities among *O. nubilalis* cDNA sequences were generated from a 1030 nt long consensus

Table 1. *Ostrinia nubilalis* midgut cDNA clones and putative derived mature serine protease properties. NA, Data not available due to assumed partial sequence

Clone name	GenBank accession	cDNA (bp)	Length (aa)	Molecular mass (kDa)	pI	Putative protease type
OnT25a	AY953059	849	233	24.81	8.57	Trypsin
OnT25b	AY953060	864	233	24.81	7.78	Trypsin
OnT25c	AY953061	858	233	24.69	7.78	Trypsin
OnT25d	AY953062	862	233	24.74	8.58	Trypsin
OnT25e	AY953063	853	233	24.74	8.27	Trypsin
T25	AY513649[a]	858	233	24.79	8.58	Trypsin
OnT23a	AY953068	821	209	22.67	5.95	Trypsin
T23	AY513650[a]	975	222	23.68	6.22	Trypsin
OnT3a	AY953064	848	243	26.43	5.31	Trypsin
OnT3b	AY953065	849	243	26.41	5.78	Trypsin
OnT3c	AY953066	850	243	26.38	5.31	Trypsin
OnT3d	AY953067	850	241	25.89	4.96	Trypsin
OnT3e	AY953052	852	156[b]	16.94	6.40	Trypsin
OnC1a	AY953069	799	232[c]	NA	NA	Chymotrypsin
OnC1b	AY953053	977	238	24.84	5.71	Chymotrypsin
OnC1c	AY953054	983	238	24.71	5.48	Chymotrypsin
OnC1d	AY953055	981	238	24.71	5.21	Chymotrypsin
OnC2a	AY953056	922	230	23.66	9.98	Chymotrypsin
OnC2b	AY953057	922	230	23.66	9.98	Chymotrypsin
OnC2c	AY953058	677	203[c]	NA	NA	Chymotrypsin

[a]Li *et al*. (2005); [b]peptide truncation due to premature stop codon; [c]partial sequence.

A

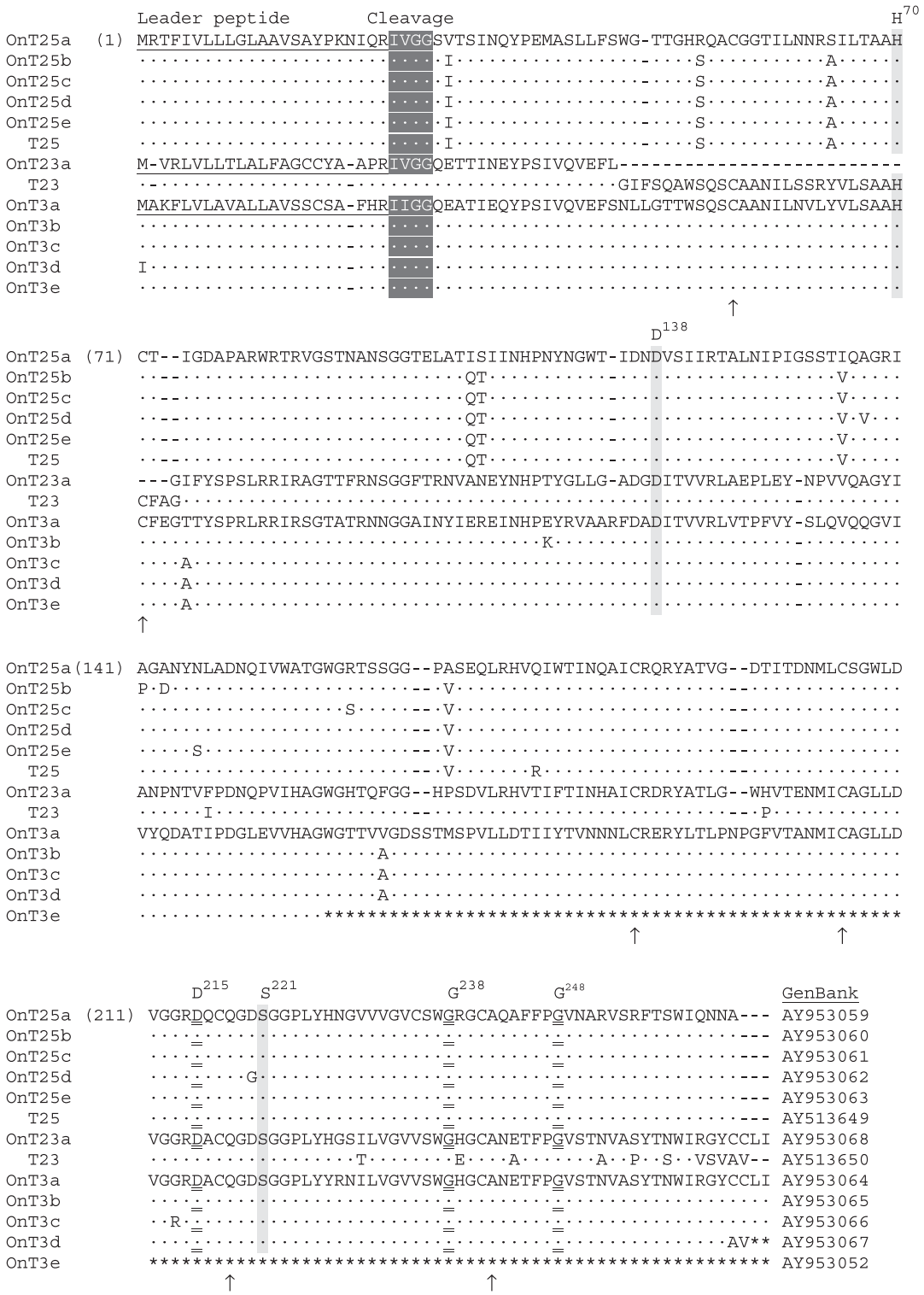
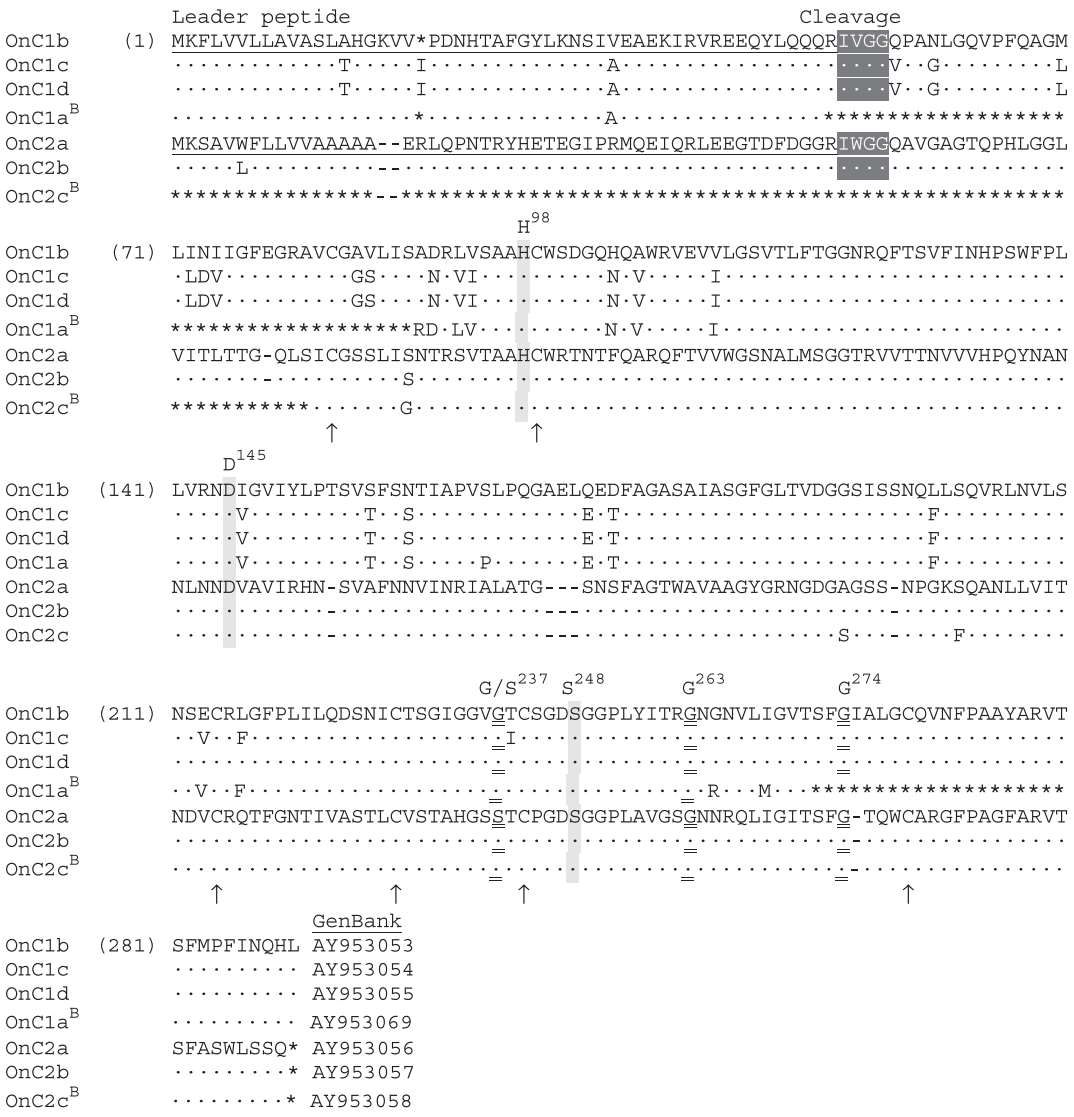


Figure 1. Trypsinogen and chymotrypsinogen-like protease sequences derived from *Ostrinia nubilalis* midgut cDNAs. (A) Alignment of 11 trypsinogen-like protease sequences. Active site residues His[70], Asp[138], and Ser [221] (highlighted), three disulphide bonds comprised on Cys residues (↑), and substrate specificity-determining residues Asp[215], Gly[238], and Gly[248] (double underlined). (B) Alignment of 7 chymotrypsinogen-like protease sequences. Active site residues His[98], Asp[145], and Ser [248], disulphide bonds comprised of three paired Cys residues, and substrate specificity determining residues Gly/Ser[238], Gly[263], and Gly[274]. For both alignments, each · indicates an identical residue to its uppermost sequence within each serine protease group, – indicates an alignment gap, and * is a residue deletion. Sequences T25 (AY513649) and T23 (AY513650) are from Li *et al.* (2005).

B



B: partial sequence.

Figure 1. *Continued*

cDNA alignment and showed five groups of serine proteases may exist (Table 2); three *O. nubilalis* trypsin-like (OnT25, OnT23, and OnT3) and two chymotrypsin-like transcript groups (OnC1 and OnC2). Five OnT25 transcripts shared ≥ 97% sequence similarity, and five OnT3 transcripts shared ≥ 88% similarity. A single group OnT23 cDNA showed 65% similarity with OnT25 and 71–73% similarity to the OnT3 class of trypsin cDNAs, but shared 83% cDNA similarity to the *O. nubilalis* trypsin T23 that was implicated in Dipel® resistance traits (Li *et al*., 2005; Table 2). Similarly, two chymotrypsin-like transcripts (OnC1 and OnC2) showed high intragroup similarities, ≥ 85% among OnC1 and ≥ 75% among OnC2 groups. Because cDNA was isolated from a pooled sample containing midgut tissue from multiple individuals, group OnT25, OnT23, OnT3, OnC1, and OnC2 each may contain different alleles from the same locus. Alternatively, each group might have representatives of different highly related genes (different loci) that are in early phase of differentiation (Zhu *et al*., 2005). This issue was addressed using Mendelian inheritance data (discussed later). Complementary DNAs resembling the 1477 nt T2 transcript (Li *et al*., 2005) were not observed in this study, and Li *et al*. (2005) did not describe OnT3-like cDNAs. Reason for difference in transcripts isolated between the two studies might be variance in transcript levels due to different genetic background of colonies used, mutation or

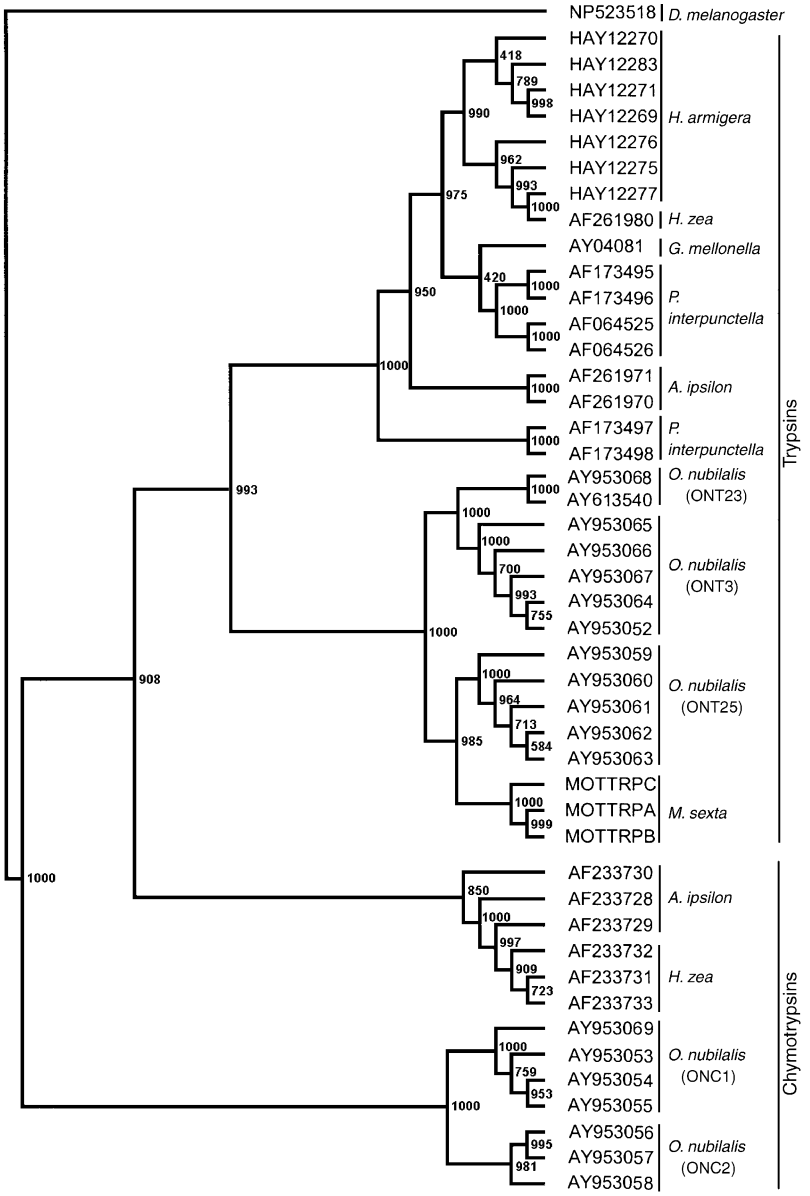


Figure 2. Ledipteran trypsinogen and chymotrypsinogen peptide sequence phylogeny using parsimony and incorporating 1 000 bootstrap iterations. GenBank accessions for 18 *Ostrinia nubilalis* serine protease peptides (ONT25, OnT23, ONT3, ONC1, and ONC2) are indicated. Additional peptides are from *Agrotis ipsilon* (AF233728, AF233729, AF233730, AF261970, AF261971), *Helicoverpa zea* (AF233731, AF233732, AF233733, AF261980), *Galleria mellonella* (AY04081), *Helicoverpa armigera* (HAY12269, HAY12270, HAY12271, HAY12275, HAY12276, HAY12277, HAY12283), *Manduca sexta* (MOTTRYPA, MOTTRYPB, MOTTRYPC), and *Plodia interpunctella* (AF173495, AF173496, AF064525, AF064526, AF173497, AF173498).

random genetic drift in the highly inbred KS-SC colony and isoline (44 and 47 generations in culture, respectively), or difference in cDNA isolation methods used.

Encoded peptides are translated as inactive zymogens that require enzymatic or autocatalytic activation (Graf *et al*., 1998). Thus all 16 derived alkaline serine proteases are zymogens, trypsinogen or chymotrypsinogen (obtained from full-length open reading frames only) that are excreted into the gut lumen prior to activation. Cytoplasmic localization and excretion of all derived peptides was predicted by PSORTII (Probability ≥ 94.1; http://www.psort.org/) using methods by Reinhardt & Hubbard (1998). Protease activation involves removal of N-terminal leader peptide regions. Previously, Bernardi *et al*. (1996) purified a 24.65 kDa trypsin-like protease with 20 residue N-terminal sequence IVGGS[X_{15}], which starts at position 24 of the derived ONT25a propeptide. The Bernardi *et al*. (1996) data suggest *O. nubilalis* ONT25 and ONT23 trypsinogens are cleaved preceding a conserved IVGG signal that releases a 23 residue leader peptide rich in positively charged amino acids (Peterson *et al*., 1994; Brown *et al*., 1997; Zhu *et al*., 2000; Li *et al*., 2005). Analogously, cleavage preceding an IIGG peptide sequence may result in a mature active ONT3 (Fig. 1A), releasing a 22-residue leader peptide. *O. nubilalis* chymotrypsinogen leader peptides are longer compared to trypsinogen counterparts. Mature ONC1 and ONC2 chymotrypsin peptides might be, respectively, formed by cleavage prior to IVGG and IWGG sequences (Fig. 1B),

Table 2. Pairwise similarity matrix of *O. nubilalis* midgut expressed serine protease cDNA sequences. Three groups of trypsin-like (OnT25, OnT23, and OnT3) and two groups of chymotrypsin-like proteases (OnC1 and OnC2) are indicated. Intraspecific cDNA sequence similarities are highlighted

	T25	OnT25a	OnT25b	OnT25c	OnT25d	OnT25e	OnT23a	T23	OnT3a	OnT3b	OnT3c	OnT3d	OnT3e	OnC1a	OnC1b	OnC1c	OnC1d	OnC2a	OnC2b	OnC2c
T25	–	98	99	99	99	99	65	68	63	63	63	63	64	45	49	50	50	49	49	53
OnT25a		–	97	97	97	98	65	68	63	63	63	64	64	45	49	50	49	49	49	53
OnT25b			–	99	99	99	65	68	63	63	63	64	64	45	49	49	49	49	49	53
OnT25c	OnT25 group			–	99	99	65	68	63	63	63	64	64	45	49	49	49	49	49	53
OnT25d					–	99	65	68	63	63	63	64	64	45	49	49	49	49	49	53
OnT25e						–	65	68	63	63	63	64	64	45	49	49	49	49	49	53
OnT23a							–	83	73	73	73	71	71	46	45	44	44	34	34	41
T23	OnT23 group							–	74	74	88	97	96	36	40	40	46	46	46	51
OnT3a									–	98	89	96	96	36	40	40	40	37	37	36
OnT3b										–	–	96	96	36	40	40	40	37	36	39
OnT3c											–	90	91	32	36	36	36	32	32	40
OnT3d	OnT3 group											–	98	36	40	40	40	38	37	41
OnT3e													–	36	40	40	40	38	38	40
OnC1a														–	85	87	86	51	51	56
OnC1b															–	99	94	55	55	46
OnC1c	OnC1 group															–	99	55	55	46
OnC1d																	–	55	55	46
OnC2a																		–	98	76
OnC2b																			–	75
OnC2c	OnC2 group																			–

which was analogous to predictions from *M. sexta* (Peterson *et al*., 1994), and *Agrotis ipsilon* and *Helicoverpa zea* (Mazumdar-Leighton & Broadway, 2001).

Following cleavage, mature active enzymes are predicted to have molecular weights of 24.69–24.81 (ONT25), 22.67 or 24.79 (ONT23), 25.89–26.43 (ONT3), 24.71 or 24.84 (ONC1), and 23.66 kDa (ONC2; Table 1). Li *et al*. (2004) showed two soluble trypsin-like peptides (240, 85, and 34 kDa) and two soluble chymotrypsin-like peptides (53 and 29 kDa) from *O. nubilalis* midgut, suggesting our cDNA sampling is representative of the low molecular weight range of enzymes. Differences in estimated molecular weights between our study and larger molecular weights from Li *et al*. (2004) might be influenced by post-translational modifications that retard peptide migration during electrophoresis. Zymogen analysis with CRY1Ab as substrate refined estimates of toxin degrading enzyme mass to 55, 38, 28 and 23 kDa (Li *et al*., 2004).

Catalytically active residues of serine proteases are referred to as the catalytic triad (Botos *et al*., 2000), and substrate-specificity determined by residues near the carboxy-terminus (Krem *et al*., 1999). Conserved trypsin-like protease residues His^{69}, Asp^{138} and Ser^{221} (catalytic triad), three paired Cys that may form disulphide bonds, and three substrate specificity determining residues, Asp^{215}, Gly^{238} and Gly^{248} were encoded by OnT25, OnT3 cDNAs, and trypsin T23 (Li *et al*., 2005; Fig. 1A).

A single transcript, OnT23a, had an internal deletion that omitted the codon for His^{70}, suggesting a non-functional enzyme (Fig. 1A). Our OnT23a transcript cDNA was 83% similar to that of *O. nubilalis* trypsin T23 (AY513650) that did encoded a catalytic His^{70}. The observed RT-PCR product length using OnT23-F and OnT23-R primers did not correspond to size predicted from the OnT23a transcript (226 bp), but was similar to that from the T23 transcript (313 bp). Experimental results suggest amino acids 45–73, including His^{70}, likely was present in expressed OnT23 alleles sampled in this study (Fig. 3). The OnT23a sequence may be a sequencing artifact, rare mutant, or unexpressed allele variation in individuals that were sampled.

Chymotrypsin-like peptides ONC1 and ONC2 showed invariable His^{98}, Asp^{145} and Ser^{242} (catalytic triad), and six Cys for disulphide bond formation (↑; Fig. 1B). First residue of the chymotrypsin substrate specificity-determining domain was variable (Gly/Ser^{238}), but remaining Gly^{263} and Gly^{274} were fixed among *O. nubilalis* sequences. All members of cDNA group OnC1 encoded Gly^{238}, while all ONC2 chymotrypsins are predicted to have Ser^{238}. Analogous variation between Gly/Ser was observed between *A. ipsilon* and *H. zea* (Mazumdar-Leighton & Broadway, 2001), and might be involved in substrate specificity differences (Botos *et al*., 2000). No *O. nubilalis* serine protease group showed elastase-like valine or threonine in the latter specificity determining residues.

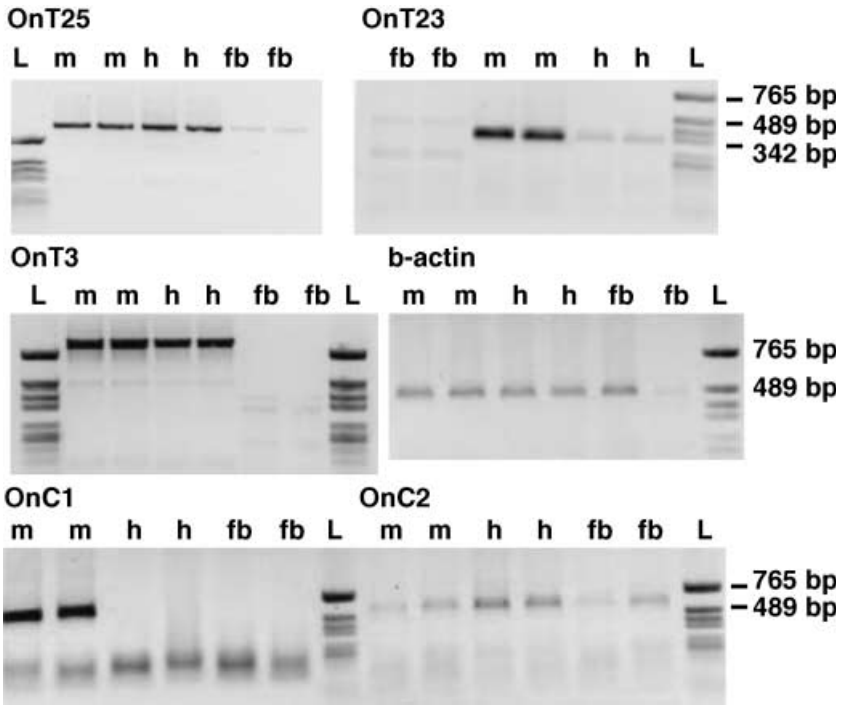


Figure 3. *Ostrinia nubilalis* trypsin- (OnT25, OnT23, and OnT3) and chymotrypsin-like (OnC1 and OnC2) transcripts detected by reverse transcriptase (RT)-PCR. Total RNA samples: m, midgut; h, head; fb, fat body. L, pGEM5Zf(+) *Msp*I digest (765, 489, 404, 342, 242, 191, 147 and 110 bp).

Trypsin- and chymotrypsin-like protease phylogeny

Trypsins and chymotrypsins may have differentiated from a single common ancestral gene (Neurath *et al.*, 1967), and the following phylogeny was used to derive inter- and intraspecific relationship among serine proteases. Peptide sequences derived from 18 variant *O. nubilalis* serine-like protease cDNAs were placed into a phylogeny with 26 midgut protease-like peptides from Lepidoptera (21 trypsin-like and 6 chymotrypsin-like). Two lineages (trypsin- and chymotrypsin-like) were indicated from parsimony tree construction, with the separation strongly supported (993 of 1000 bootstrap iterations; Fig. 2). Bootstrap branch support was > 71.3% of 1000 bootstraps for all but two internal nodes. GenBank accession HAY12270 was separated from other *Helicoverpa armigera* trypsins in 418 of 1000 random trees and the node separating *Galleria mellonella* (AY04081) from *Plodia interpunctella* trypsins was supported by 420 of 1000 bootstrap iterations, suggesting indeterminate genealogical relationships at those nodes. Parsimony analysis clustered 13 *O. nubilalis* peptides ONT25, ONT23, and ONT3 into three phylogenetic groups nested within trypsin-like proteases. OnT25 derived peptides may show common ancestry to *M. sexta* trypsins (Lepidoptera: Sphingidae; MOTTPPA, B, and C). ONT23 and ONT3 peptides grouped separate from the ONT25 groups, but show a common ancestral gene. The phylogeny indicated ONT23 (and AY513650) were more highly related to ONT3 compared to ONT25 group peptides, which supported conclusions drawn from cDNA sequence similarities (Table 2). Trypsins from the pyralid *P. interpunctella* did not group near those from *O. nubilalis*, suggesting high levels of peptide variation outside of functional residues.

Derived peptides ONC1 and ONC2 were separated from heliothine (*H. zea* and *A. ipsilon*) chymotrypsins by 100% of reconstructed trees, and ONC1 and ONC2 peptides comprised two differentiated groups (1000 of 1000 bootstraps). The *O. nubilalis* chymotrypsin-like proteases may form a unique clade or lineage. Alternatively, the few number of isolated lepidopteran chymotrypsin genes might contribute to lack of close phylogenetic relatedness. Incorporation of additional yet uncharacterized chymotrypsin sequences are required to fully resolve the phylogeny.

Trypsin- and chymotrypsin-like protease transcription

Reverse transcriptase (RT)-PCR indicated *O. nubilalis* trypsin and chymotrypsin-like transcripts for OnT25, OnT23, OnT3, OnC1, and OnC2 are present in midgut tissue (Fig. 3). Members of serine protease gene families are expressed in midgut epithelial cells and function as peptide digesting enzymes (Peterson *et al.*, 1994; Brown *et al.*, 1997). The OnT25, OnT23, OnT3, and OnC2 transcripts also were present in total RNA extracted from *O. nubilalis* head tissues, and may indicate salivary gland expression (note: OnT23 head expression is very low; Fig. 3). Serine proteases in salivary juices may function in digestion prior to entry into the alimentary canal (Cohen, 1998). The Hessian fly expresses two chymotrypsins, MDP1

and MDP2, and two trypsins, MDP3 and MDP4, in gut and salivary tissue (Zhu *et al.*, 2005), whereas a third trypsin, MDP5, was specifically expressed in the gut. The trypsin transcript HaP1 from *H. armigera* also was expressed only by midgut tissue. Only OnC1 was specifically expressed by the midgut, and OnT23 only showed low expression in head tissue compared to midgut. Additionally, RT-PCR detected OnT25- and OnC2-like transcripts in *O. nubilalis* fat body tissues (Fig. 3), suggesting lepidopteran serine protease genes have different degrees of tissue specificity. The β-actin control showed equal RT-PCR amplified band intensities for all tissue samples, except the second replicate of total RNA derived from fat body tissue. Decreased band intensity likely was due to experimental error (reaction or gel loading) as opposed to transcript level, since products OnT25, OnT23, OnT3, OnC1 and OnC2 were of equivalent intensity as the first replicate of the fat body tissue sample. All RT-PCR product lengths were as predicted from the cDNA sequence (Table 3), except OnT23a (explanation above).

Genomic markers

Polymorphic genomic markers were developed for the *O. nubilalis* genes OnT23, OnT3, OnC1 and OnC2, and used to investigate Mendelian inheritance pattern and show single locus specificity. Intraspecific alignment of *O. nubilalis* protease cDNAs identified 17, 14, 22, 32 and 10 single nucleotide polymorphisms (SNPs) among OnT25, OnT23, OnT3, OnC1 and OnC2 cDNAs, respectively (alignments not shown). Single genomic DNA fragments containing respective trypsin or chymotrypsin gene were PCR amplified using same primers as in RT-PCR (Table 3). Some SNPs within amplified regions were detected by PCR-RFLP assays. Polymorphic OnT23 and OnT3 fragment patterns resulted when digested with *Hae*III, and similarly OnC1 with *Hae*III or *Hin*fI, and OnC2 with *Cfo*I. No variation was observed among alleles at the OnT25 locus, and was not used in subsequent pedigree analysis. Polymorphism of PCR fragments may result from the coamplification of more than one locus or represent allelic variation at a single locus, and the latter can be established by observation of Mendelian inheritance patterns.

OnT3 *Hae*III and OnC2 *Cfo*I markers were polymorphic within pedigree Ped10B, and OnT23, OnT3, and OnC1 *Hae*III variation was observed within Ped24a. Parents and F_2 offspring genotypes were determined at all polymorphic loci, and data used to test for Mendelian inheritance of observed alleles. Chi-square tests (χ^2) determined that the allelic distribution among F_2 individuals did not deviate significantly from expected Mendelian ratios as determined from parental genotypes, except for alleles of the OnC2 locus (Table 4). Deviation likely resulted from segregation of two null alleles at the locus, coamplification of alleles from ≥ 1 locus, or non-random mating among F_1 progeny. Therefore, the *O. nubilalis* OnT23 *Hae*III, OnT3 *Hae*III, and OnC1 *Hae*III PCR-RFLP defined alleles are inherited in a Mendelian manor within the pedigrees tested (Table 4). This suggests OnT3, OnC1 and the Dipel® resistance-associated OnT23 gene is locus-specific and appropriate for use in genomic linkage mapping or phenotypic association studies.

Molecular screening is increasingly being incorporated into resistance diagnostics (Brogdon & McAllister, 1998), and comparative studies showed association of three cadherin alleles (r1, r2 and r3) with *Pectinophora gossypiella* Bt resistance traits (Morin *et al.*, 2003). Assays also were developed to screen *O. nubilalis* cadherin allele (Coates *et al.*, 2005). Codominant genetic markers used must show Mendelian inheritance and be devoid of non-PCR amplifying null alleles for use in phenotypic linkage or association mapping studies (Pemberton *et al.*, 1995). The T23 *Hae*III marker did show Mendelian inheritance pattern (Table 4). The marker will be applied in future studies involving inheritance of Bt toxin resistance phenotypes, and used in linkage mapping or studies to associate cosegregation of alleles with bioassayed *O. nubilalis* Bt toxin resistance phenotypes.

Table 3. *Ostrinia nubilalis* trypsin- and chymotrypsin-like serine protease gene primers used for reverse transcriptase (RT)-PCR and genomic DNA amplification (PCR) assays. RT-PCR sizes are calculated from cDNA sequence data

Primer name	Primer sequence (5′ to 3′)	Annealing temperatures			RT-PCR size (bp)
		RT 1st	RT 2nd	PCR	
OnT25-F	CAAAAATGCGTACCTTCATCGTTC	56 °C	56 °C	62 °C	812
OnT25-R	ATCATCAGCATCCGTAACGTG				
OnT23-F	CGCAGCGCCCAGAATCGT	54 °C	58 °C	60 °C	313
OnT23-R	CAGCCTCACAACGGTGATGTC				
OnT3-F	GCGATATGGCGAAATTCTTGG	50 °C	50 °C	62 °C	800
OnT3-R	TATTAAACAGCAGTAGCCGCG				
OnC1-F	CATTATGAAGTTCCTGGTCGTCCT	50 °C	50 °C	60 °C	533
OnC1-R1	CGAAGGTCTCCTCCAGCTCA				
OnC2-F2	ACCGGCTCGAACAGCTTCG	56 °C	56 °C	62 °C	463
OnC2-R	GCATCAATCATTGGCTGCTCAA				

Table 4. Test of Mendelian inheritance of trypsin and chymotrypsin alleles in *Ostrinia nubilalis* pedigrees Ped10b and Ped24a. PCR amplified genomic DNA fragments from OnT23, OnT3, and OnC1 genes were digested with *Hae*III, and OnC2 with *Cfo*I. Genotypic ratio among F2 offspring was compared to the expected Mendelian proportions based on parental genotypes. Significant allele frequency departures from Mendelian expectation were tested by χ^2 analysis (significance at < 0.05). Approximate sizes of observed PCR-RFLP fragments for alleles in pedigrees that showed Mendelian inheritance, and predicted but unobserved fragment sizes are within parentheses. NP, no polymorphism

Pedigree		Parental genotype	Observed F_2 PCR-RFLP genotypes	Expected F2 ratio	χ^2	*P*-value (d.f.)
Ped10b	OnT3	♂A11 ♀A12	A11 (28): A12 (12): A22 (4)	9 : 6 : 1	2.22	0.3292 (2)
	OnC1	♂E11 ♀E11	NP			
	OnC2	♂C33 ♀C34	C33 (8): C34 (34): C44 (0)	9 : 6 : 1	34.11	< 0.0001 (2)
Ped24a	OnT23	♂H12 ♀H23	H11 (2): H12 (8): H22 (12): H23 (12): H13 (1): H33 (5)	1 : 4 : 4 : 4 : 2 : 1	7.00	0.2206 (5)
	OnT3	♂A13 ♀A33	A33 (27): A13 (12): A11 (5)	9 : 6 : 1	3.27	0.1947 (2)
	OnC1	♂E22 ♀E11	E11 (10): E12 (23): E22 (15)	1 : 2 : 1	1.25	0.5698 (2)
	OnC2	♂H33 ♀H33	NP			

Gene	Allele	Fragments
OnT3	A1	470 and 460 bp
	A3	340, 300, 150 and 140
OnT23	H1	1230
	H2	780 and 490
	H2	765, 490 and (15)
OnC1	E1	740
	E2	490 and 240

Experimental procedures

RNA sample tissue

Bivoltine Z-pheromone strain *O. nubilalis* adults were field collected at the Iowa State University Uthe Farm (Ames, IA) and maintained in culture at the USDA-ARS Corn Insects and Crop Genetics Research Unit (CICGRU) facilities in Ames, IA for three to five generations. Larvae were reared on a semi-meridic diet (Guthrie, 1987), midguts dissected from six 5th instars, samples pooled, and ground in liquid nitrogen with a mortar and pestle. Additionally, fat body, midgut, and head were dissected from two 5th instars, tissues kept separate, and ground to powder in liquid nitrogen. RNeasy extraction kits (Qiagen, Valencia, CA, USA) were used according to manufacturer instructions to obtain total cellular RNA from 10 mg of ground *O. nubilalis* tissues. The extracts were quantified by absorption at 260 nm (A_{260}), diluted to 50 ng/μl with nuclease free (0.1% diethylpyrocarbonate-treated) water, and used immediately.

Complementary DNA (cDNA) synthesis and cloning

First strand cDNA synthesis used 0.5 μM of a poly(T) adapter (PT-AD; 5′-GGT GTA ATA CGA CGG CCT GGA ATT CTT TTT TTT TTT TTT TTT T-3′), 2 μg of total RNA, 40 U of AMV reverse transcriptase (Promega, Madison, WI, USA) in a 20 μl reaction that was incubated at 42 °C for 1 h on a PTC-100 thermocycler (MJ Research, Watertown, MA, USA). Subsequent PCR used 2 μl first strand cDNA as template, 5 pmol degenerate primer 5′-CAG GGT GAC TCY GGC GGY C-3′ (corresponding to the conserved QGDSGGP peptide sequence; Mazumdar-Leighton *et al.*, 2000), 5 pmol poly(T) adapter core primer 1 (PT-Adc1; 5′-GTG TAA TAC GAC GGC CTG G-3′), 0.9 U *Tli* polymerase and 0.1 U *Taq* polymerase (both Promega), 50 μM dNTPs, 1.5 mM $MgCl_2$, and 2.5 μl of Thermopolymerase Buffer (Promega) in a 25 μl reaction. A PTC-100 thermocycler (MJ Research) performed 35 cycles of 95 °C for 30 s, 60 °C for 20 s, and 72 °C for 30 s, followed by 72 °C for 10 min. Amplification success was confirmed and fragment sizes estimated by electrophoresis of 10 μl PCR product on a 10 cm 1.5% agarose 1× TBE (Tris-Borate-EDTA, pH 8.0) gel containing 0.5 μg/ml ethidium bromide. PCR products were ligated using the pGEM-T easy cloning system (Promega), incubated overnight at 4 °C, and used to transform 80 μl of *E. coli* SURE (Stratagene, La Jolla, CA, USA) by electroporation. Sixteen insert positive plasmids containing 3′ RACE products were identified, and each sequenced using 10 μl DTCS Quickstart DNA sequencing reactions (Beckman-Coulter, Fullerton, CA, USA) according to manufacturer instructions with 1.6 pmol T7 primer. Primer extension products were purified by ethanol precipitation, suspended in 40 μl deionized formamide, and separated on a CEQ 8000 Genetic Analysis System (Beckman-Coulter) with method LFR-1 (denature: 90 °C for 120 s; inject: 2.0 kV for 15 s; and separated: 4.2 kV for 85 min in a 50 °C capillary). Sequence data was used to design transcript-specific reverse primers; OnT25–5pR (5′-ATC ATC AGC ATC CGT AAC GTG-3′), OnT23–5pR (5′-TTG ACA CCA GGG AAG AAA GCG-3′), OnT3–5pR (5′-TAT TAA ACA GCA GTA GCC GCG-3′), OnC1–5pR (5′-GAT GTT GTT TAT AGG TGC TGG TTG A-3′), and OnC2–5pR (5′-GCA ATC AAT CAT TGG CTG CTC AA-3′).

First Choice RNA Ligase-Mediated (RLM) RACE kits (Ambion, Austin, TX, USA) were used to recover 5′ cDNA ends for each transcript in accordance with manufacturer's instructions. RLM-RACE template was generated in reactions that used 1 μg of total RNA, and transcript-specific reverse transcriptase (RT)-PCR products generated separately from primers located in the 3′-UTR in conjunction with the RLM-RACE 5′ outer primer. Subsequent 25 μl hot start PCR used 1.0 U *Tli* polymerase (Promega), 200 μM dNTPs, 1.5 mM $MgCl_2$, and 2.5 μl of 10 × thermal polymerase buffer (Promega), 1 μl RLM mRNA template, 5 pmol transcript-specific reverse primer and 1 μl RLM-RACE 5′ adapter outer primer (Ambion; 5′-GCT GAT GGC GATG AAT GAA CAC-3′). A PTC-100 thermocycler (MJ Research) cycled 40 times at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 m. RT-PCR products were ligated and used to transform the *E. coli* SURE host strain. Forty clones

carrying plasmid inserts containing 5′ RACE products were identified (nine OnT25, one OnT23, 10 OnT3, 10 OnC1 and 10 OnC2), and DNA sequence was obtained as described previously.

Trypsin- and chymotrypsin-like protease phylogeny

Twenty six lepidopteran trypsin and chymotrypsin peptide sequences were obtained from GenBank, and used to construct a 318 amino acid consensus alignment after inclusion of derived amino acid sequence from 18 unique cDNAs encountered in the present study. All subsequent manipulations use the PHYLIP software package (Felsenstein, 1989). One thousand bootstrap resampling steps were produced by the SeqBoot program, parsimony trees were generated using ProtPars, a strict consensus tree was estimated from all possible phylogenies with CONSENSE, and was viewed using TreeView (Page, 1996).

Trypsin- and chymotrypsin-like protease expression

Total RNA extracted from fat body, midgut, and head of 5th instar *O. nubilalis* was subjected to RT-PCR analysis. Individual first strand cDNA synthesis reactions used 250 ng total RNA template, 10 pmol reverse primer (Table 3) or β-actin-R (5′-GAC AAC GGC TCC GGT ATG T-3′; controls only), 2.5 U *Tth* polymerase (Promega), 100 μM dNTPs, 2.5 mM $MnCl_2$, and 2.0 μl of 10 × *Tth* reverse transcriptase buffer (Promega) in a 10 μl reaction. A PTC-100 thermocycler (MJ Research) performed a primer extension cycle of 85 °C for 1 min, 50 or 56 °C for 1 min, and 72 °C for 20 m. A 4.0 μl aliquot of 1st strand cDNA synthesis product was mixed with 1.6 μl chelate buffer (Promega), 10 pmol of primer forward, or β-actin-F (5′-CCT TCG TAG ATA GGG ACG GT-3′; controls only), and $MgCl_2$ concentration adjusted to 2.0 mM in a 20 μl final volume. PCR reactions were carried out on a PTC-100 thermocycler (MJ Research) using 40 cycles of 95 °C for 30 s, 50 or 56 °C for 30 s (Table 3), and 72 °C for 1 min. RT-PCR products (20 μl) were separated on a 10 cm 1.0% agarose 1 × Tris-borate EDTA gel containing 0.5 μg/ml ethidium bromide, and digital images taken under UV illumination on a Bio-Rad ChemiDoc System (Bio-Rad, Hercules, CA, USA).

Genomic marker development

Mendelian inheritance of PCR-RFLP markers was evaluated by observing allele segregation in pedigrees Ped10b and Ped24a. Two paired matings of *O. nubilalis* adults from the USDA-ARS, CICGRU laboratory colony established F_1 families. The F_1 progeny were sib-mated and resultant F_2 larvae reared on a semimeridic diet (Guthrie, 1987). DNA was extracted according to Coates & Hellmich (2003), and samples stored at –20 °C prior to use.

Original cDNA sequences were aligned using AlignX (Informax, San Francisco, CA, USA). Polymorphic restriction endonuclease cleavage sites were identified. SNPs were detected by PCR-RFLP, with amplification of OnT23, OnC1 and OnC2 gene fragments using primers in Table 3. OnT25 and OnT3 used different reverse primers, OnT25-R2 (5′-GAT GTC AAG GGC GGT TCG GA-3′) and OnT3 (5′-GTA CAC GAA GGGGGT GAC CA-3′), respectively. PCR reactions (10 μl) used 0.25 U *Taq* polymerase (Promega), 200 μM dNTPs, 2.5 mM $MgCl_2$, and 1.0 μl of 10 × thermal polymerase buffer (Promega), 100 ng of genomic DNA, and 2 pmol of each forward and reverse primer. The OnT25 genome fragment was digested with *Rsa*I or *Hae*III. OnT23, OnT3 and OnC1 were digested with *Hae*III, and OnC2 with *Cfo*I. All endonucleases were purchased from Promega. PCR-RFLP reactions included 5.0 μl of appropriate PCR product, 2.5 μl 10 × Buffer, 0.1 mg/μl BSA, and 0.25 U of enzyme in 25 μl. Reactions were incubated at 37 °C or 60 °C (*Taq*I) for 14 h. Entire PCR-RFLP reactions were loaded on to 10 cm 2% 1 × TBE agarose gels containing 0.5 μg/ml ethidium bromide. Samples were separated at 100 V for 1 h, and images captured under UV illumination on a ChemiDoc System (Bio-Rad). Fragment sizes are estimated using Gel-Pro Analyser (Media Cybernetics, Silver Springs, MD) by comparison to a pGEM5Zf(+) *Msp*I digest ladder (765, 489, 404, 342, 242, 191, 147, and 110 bp). Chi-square tests (χ^2) measured goodness-of-fit between observed allelic distributions among F_2 and Mendelian ratios expected from parental genotypes.

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